UNIVERSITI PUTRA MALAYSIA

AGROBACTERIUM-MEDIATED TRANSFORMATION OF OIL PALM (*ELAEIS GUINEENSIS* JACQ.) SUSPENSION CULTURE

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By

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Chairman : Associate Prof Dr. K. Harikrishna

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The life cycle of flowering plants in general can be divided into two growth phases: vegetative and reproductive. The reproductive phase can be subdivided into the development of the inflorescence meristem and floral meristems. Control of flowering and the regulation of plant architecture has been thoroughly investigated in a number of well-studied dicot plants such as *Arabidopsis*, *Antirrhinum*, tomato and tobacco. However, in monocot plants, molecular information related to plant reproduction is still limited. In

Arabidopsis, the *Terminal Flowering* 1(*TFL*1) gene, *LEAFY*, and the target genes of *CONSTANS* (*CO*) including the *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO* 1 (*SOC*1) genes, have a major role in promoting flowering and thus controlling flowering time.

To investigate the regulation of meristem identity as well as the control of floral transition in oil palm, we transferred genes *pCAMBIA/TFL1*, *pCAMBIA/JIT60*, *pCAMBIA/LFY*, *pGA/LFY* and *pCAMBIA/SOC1* into oil palm embryogenic callus. This present study focuses on the optimization of *Agrobacterium*-mediated transformation and the analysis of transgenic plants. The objective of this study was also to clone the putative oil palm (*OPSOC*1 and *OPLFY*) genes into a plasmid binary vector system, so as to transform these genes into oil palm embryogenic callus and to determine their effect on expression driven by a cauliflower mosaic virus (CaMV) 35S promoter in oil palm.

The success of gene delivery using *Agrobacterium* into the plant genome is often based on several factors including temperature used during co-cultivation, the binary vector and promoters used, and the plant genotype itself. The most important factors contributing to the success of T-DNA transfer is the type of plant material used. We used embryogenic suspension cells as starting material for the transformation of oil palm because of the large numbers of totipotent cells found in these cultures.

The results of the present study show that, embryogenic suspension cells are suitable for *Agrobacterium*-mediated transformation of oil palm. Stable *in vitro* transgenic oil palm plants have been successfully produced through *Agrobacterium*-mediated DNA

delivery within 7-8 months after co-cultivation. A total of 101 transgenic plants, each derived from single embryogenic clusters were obtained from 5563 *Agrobacterium* infected embryogenic clusters from four oil palm lines. The overall transformation frequency was about 1.82%, as it is an average frequency across four parameters (preculture of the embryogenic callus, the effect of temperature during co-cultivation, the effect of binary vectors and the effects of genotype on transformation rate), it is likely that a higher transformation frequency could be obtained routinely under a more optimal set of conditions. Analysis using PCR and Southern Blotting confirmed that the T-DNA was successfully integrated into the oil palm genome.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

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Kitaran hidup tumbuhan berbunga secara amnya boleh dibahagikan kepada 2 peringkat pertumbuhan : vegetatif dan reproduktif. Fasa reproduktif pula boleh dibahagikan kepada pembentukan meristem infloresen dan meristem bunga. Pengawalan pembungaan dan rupabentuk tumbuhan telah dikaji secara mendalam pada beberapa tumbuhan dikot seperti *Arabidopsis*, *Antirrhinum*, tomato dan tembakau. Walaubagaimanapun, pada tumbuhan monocot, maklumat molekular dalam penghasilan tumbuhan masih terhad. Di dalam tumbuhan *Arabidopsis*, gen "*Terminal Fowering* 1 (*TFL*1)", "*LEAFY*" dan gen sasaran "*CONSTANS* (*CO*) " meliputi gen "*FLOWERING*

LOCUS T (FT)" dan gen "*SUPPRESSOR OF OVEREXPRESSION CO 1 (SOC 1)*" memainkan peranan utama dalam mempromosi dan mengawal waktu pembungaan.

Dalam mengkaji perubahan identiti meristem dan pangawalan perubahan pembungaan di dalam kelapa sawit, kami memindahkan gen-gen *pCAMBIA/TFL1*, *pCAMBIA/JIT60*, *pCAMBIA/LFY*, *pGA/LFY* dan *pCAMBIA/SOC1* ke dalam sel embriogenik kelapa sawit. Penyelidikan ini memfokuskan kepada pengoptimaan transformasi menggunakan kaedah *Agrobacterium* dan penganalisaan tumbuhan transgenik. Objektif penyelidikan ini juga adalah untuk mengklon gen putatif *SOC1* dan *LFY* ke dalam vektor plasmid bersistem binari, mentransformasikan gen tersebut ke dalam sel embriogenik kelapa sawit dan seterusnya mengkaji ekspresi gen tersebut dengan menggunakan promoter CaMV35S.

Kejayaan pemindahan gen menggunakan *Agrobacterium* ke dalam genom tumbuhan sering bergantung kepada beberapa faktor termasuk suhu yang digunakan semasa ko-kultivasi, vektor binari dan promoter yang digunakan, dan juga jenis tumbuhan itu sendiri. Faktor yang paling penting yang boleh menghasilkan kejayaan untuk pemindahan T-DNA ke dalam genom tumbuhan adalah jenis bahan tumbuhan yang digunakan. Kami menggunakan sel embriogenik kelapa sawit terampai sebagai bahan untuk proses transformasi kerana kandungan sel 'totipotent' yang terdapat pada bahan tersebut.

Keputusan daripada penyelidikan menunjukkan, ampaian sel embriogenik adalah sesuai untuk transformasi menggunakan *Agrobacterium.* Tumbuhan kelapa sawit transgenik yang stabil, di dalam tabung uji telah dihasilkan melalui kaedah pemindahan gen menggunakan *Agrobacterium* dalam tempoh 7 hingga 8 bulan selepas ko-kultivasi. Sejumlah 101 tumbuhan transgenik yang muncul daripada sekumpulan sel embriogenik diperolehi daripada 5563 kumpulan sel embriogenik. Kadar transformasi secara keseluruhan adalah 1.82%, tetapi ini adalah berdasarkan purata penghasilan daripada rawatan yang berbeza. Penganalisaan menggunakan 'PCR' dan 'Southern Blotting' mengesahkan kemasukan T-DNA ke dalam genom tumbuhan kelapa sawit.

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I certify that an Examination Committee met on 27th June 2003 to conduct the final examination of Siti Habsah Roowi on her Master of Science thesis entitled "*Agrobacterium*-mediated transformation of Oil Palm (*Elaeis guineensis* Jacq.) Suspension Culture" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(SITI HABSAH ROOWI)

Date: 8.9.2003

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AS	acetosyringone
bp	base pair
CaMV	cauliflower mosaic virus
CIM	callus induction medium
^{o}C	degree centigrade
DNA	deoxyribonucleic acid
2,4-D	2,4-dichlorophenoxyacetic acid
DMSO	dimethyl sulfoxide
DMRT	Duncan's Multiple Range Test
EDTA	ethylenediaminetetraacetic acid
GUS	*ß*-Glucuronidase
g	gram
HPT	hygromycin phosphotransferase gene
h	hour (s)
mM	millimolar
μM	micromolar
ml	milliliter
mg	milligram (s)
MS	Murashige and Skoog
MSO	hormoneless Murashige and Skoog
NaOH	Sodium hydroxide

Nos ter	Nopaline synthase gene terminator
NPTII	Neomycin phosphotransferase gene
OD	Optical density
%	Percentage
PCR	Polymerase Chain Reaction
RNase	ribonuclease
rpm	revolution per minute
35S	35 subunit of ribosome
T-DNA	transferable-DNA
µl	microliter
v/v	volume per volume
w/v	weight per volume
X-gluc	5-bromo-4-chloro-3-indoyl-glucuronide

CHAPTER 1

INTRODUCTION

Oil palm is one of the larger members of the palm species. It has a single stem, and unlike some palms, does not produce suckers from the base. There are about 212 genera and about 2500 species of palms that grow in the tropics and subtropics. The single vegetative growing point is situated in a depression at the stem apex. The meristem is continuously active, producing a new leaf primordium about every two weeks in a mature palm. The leaf is pinnate, with the pinnae (leaflets) arranged in two or more planes on each side of the rachis. In each leaf axil produced in cycles of variable duration, are inflorescences that following pollination develop into a fruit bunch, from which mesocarp and kernel oils can be extracted.

The oil palm is a particularly important species in world trade as a major economic oil crop. It also has wide usage in the food and non-food industries. Currently, Malaysia is the largest palm oil producer in the world with 3.5 million hectares of land devoted to oil palm cultivation, representing more than 60% of the country's total cultivated agricultural land and has become the world's largest exporter of palm oil with a market share of 60.6%. However, soon Indonesia will overtake Malaysia and become the largest palm oil producer in the world. Thus, the palm oil industry in Malaysia is undergoing a challenging time.

Due to the increased demand for palm oil, increasing the oil yield is the main agenda for those involved in the oil palm industry. Research in plant genetics and breeding

has largely improved the performance of the crop, however, with conventional breeding and seed production methods the maximum potential of the selected hybrid genotypes may still not be realized. Therefore, vegetative propagation of elite hybrid oil palm via tissue culture seems to be an attractive alternative.

Gene transfer to elite germplasm is an attractive method for more rapid genetic improvement of this perennial crop. Several techniques such as direct DNA uptake using biolistic or particle gun bombardment, and vector-mediated gene delivery using *Agrobacterium tumefaciens*, have been used for oil palm transformation. However, the success of gene transfer depends on several factors, such as the use of suitable promoters and the appropriate selectable marker gene with a sensitive selection agent, the use of appropriate target tissue and development of a reproducible regeneration protocol. The lack of an efficient regeneration method is a great bottleneck in the application of transformation technology to oil palm. The regeneration of genetically transformed plants based on somatic embryogenesis could be a viable solution for the oil palm.

The aim of this thesis was to establish an efficient procedure for the stable transformation of foreign genes into oil palm via co-cultivation with *A. tumefaciens* and to study the integration and expression of foreign genes in transgenic oil palm by molecular analyses such as PCR and Southern blot hybridization. The experiments conducted in this thesis are divided into 3 parts:

1. Construction of binary transformation vector.
2. Transformation of several genes of interest into oil palm embryogenic suspension cultures.
3. Analysis of putative transformed plantlets.

CHAPTER 2

LITERATURE REVIEW

2.1 The Botany of Oil Palm

The oil palm (*Elaeis guineensis Jacq.*) is classified under the family Palmae in the order Palmales and grouped with the cocos, coraza and other genera under the tribe Cocoinae. It is a large feather palm having a solitary columnar stem with short internodes. The palm is normally monoecious with male or female, or at certain stages hermaphrodite inflorescences developed in the axil of the leaves. The fruit is a drupe, which is borne on a large compact bunch. The oil palm seed is a nut that remains after the soft oily mesocarp has been removed. It is composed of a shell or endocarp surrounding usually one kernel and sometimes up to 4 kernels. The embryo is straight and about 3mm in length. Its distal end lies opposite the germ pore separated by a thin layer of endosperm cells.

The seedling has three months to establish itself as an organism capable of photosynthesis and absorption of nutrients from the soil. The first adventitious roots are produced in a ring just above the radicle hypocotyl junction and these give rise to secondary roots before the first foliage leaf has emerged. The radicle continues to grow for about six months by which time it would have reached about 15 cm in length (Figure 1).